Development of real-time PCR assays for detection of white spot syndrome virus, yellow head virus, Taura syndrome virus, and infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp

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Abstract

Real-time PCR and real-time RT-PCR (reverse transcription-PCR) methods have been developed for shrimp viruses. Real-time PCR was developed for the detection of white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV). Real-time RT-PCR was developed to detect Taura syndrome virus (TSV) and yellow head virus (YHV). The work involves the selection of PCR (and RT-PCR) primers and TaqMan probes from the viral genomes. These primers/probes proved to be conserved among various viral isolates, and they are specific to each virus. The assay protocols have been optimized to include 300 nM of primers and 150 nM of TaqMan probe. Recombinant plasmids were constructed and cloned to use as positive controls. The sensitivity was determined to be less than 10 copies for IHHNV and WSSV, and 10-100 for YHV and TSV. This method, which has been shown to be rapid with high throughput and sensitivity, can be used to screen viruses in shrimp and other marine products that are imported to the United States as well as to those that are being exported.

Executive Summary

The goal of this project is to develop a real-time PCR (and RT-PCR) assay for each of four major shrimp viruses: white spot syndrome virus (WSSV), Taura syndrome virus (TSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), and yellow head virus (YHV). For each virus, the specific aims were: (1) selection of PCR (or RT-PCR) primers/TaqMan probes; (2) development of appropriate real-time PCR (or RT-PCR) protocols; and (3) construction and cloning of recombinant plasmids to be used as positive controls. We selected a set of primers/probe for IHHNV and for YHV, and 2 sets of primers/probes for WSSV and for TSV. The primers/probes are conserved among various geographic isolates. They also proved to be specific to each virus, and they did not react to other shrimp viruses nor to shrimp DNA (or RNA). A real-time PCR (and RT-PCR) instrument, GeneAmp 5700 sequence detection system has been purchased with non-federal funds and used to optimize the concentration of PCR primers and TaqMan probe. The highest signal can be achieved at 300 nM of primers and 150 nM of TaqMan probe.

For each virus, a recombinant plasmid inserted with a target sequence was constructed and purified to be used as both qualitative and quantitative controls. The detection limit was less than 10 copies for WSSV and IHHNV, and it ranged from 10 to100 copies for TSV and YHV. We also applied these assays to quantify the viral load in several tissue samples. Tissues of penaeid shrimp that became moribund from WSSV infection contained up to10^{10} molecules of WSSV genome per ：g of extracted DNA. Also, tissues of shrimp (*Penaeus stylirostris* and *P. vannamei*) that were highly infected with IHHNV contained more than 10^9 molecules of IHHNV genomes per ：g of DNA. The quantities of TSV were also determined in individual *P. vannamei* with acute (10^7 molecules/：g RNA) and chronic (10^5 molecules/：g RNA) infections.

This assay is rapid with a high-throughput; the results of 96 PCR (and RT-PCR) reactions can be analyzed in 2-2.5 hrs. Also, the quantitative results from the real-time PCR (and RT-PCR) are reproducible. This procedure is, therefore, highly suitable for detection and quantification of shrimp viruses.

Purpose

The recent discovery that exotic viruses remain infectious in frozen, commodity shrimp has led to serious concerns in the United States that these imported products may pose a risk to our domestic fishery and aquaculture industries. This concern is also shared by countries that import shrimp and other marine products from the United States. The lack of a rapid, sensitive, test method for specific viruses in such items poses a serious constraint to the U.S. international trade in aquaculture and fishery-related products.

Because of the potential threat to both wild and farmed populations of shrimp in U.S. waters, the government may find it prudent to prohibit the importation of shrimp from countries known to have viral epizootics. This would result in a trade barrier, estimated to be $12 billion, that will have a major economic impact in the U.S. In addition, U.S. exports are being adversely affected by very recent

regulations imposed by its trade partners. The U.S. exports a variety of aquatic products to countries that require imports to be certified free of specific viruses.

There is a urgent need to develop rapid and sensitive diagnostic methods for detection of viruses in shrimp that are being imported to, and exported from, the U.S. The objectives are to develop real-time PCR (and RT-PCR) assays for four major shrimp viruses.

Approach

Description of work:

1. Acquire a real-time PCR (and RT-PCR) detection system
2. Optimize DNA/RNA extraction protocols
3. Develop real-time PCR (and RT-PCR) assays
   - 3.1. selection of PCR primers/TaqMan probes for IHHNV and WSSV
   - 3.2. selection of RT-PCR primers/TaqMan probes for YHV and TSV
   - 3.3. construction of positive controls for IHHNV and WSSV
   - 3.4. construction and synthesis of positive controls for YHV and TSV
4. Optimize real-time PCR (and RT-PCR) protocol
5. Evaluation of sensitivity and reproducibility of real-time PCR (and RT-PCR)

Project management:

This work was carried out mainly by two postdoctoral research associates, Kathy Tang-Nelson and Stephanie Durand, under the supervision of Donald Lightner at the University of Arizona. Several lab technicians provided assistance in preparation of infected shrimp, purification of viruses, extraction of viral DNA or RNA, and cloning of positive control plasmids.

Findings

The tasks that have been accomplished are:

1. Acquire a real-time PCR system

A real-time PCR instrument, GeneAmp 5700 (Applied Biosystems, Foster City, CA, USA), was purchased with non-federal funds and installed in our laboratory at the University of Arizona. This instrument provides a detection sensitivity of less than 10 copies with a 7-8 log linear relationship in quantitative studies. It uses a 96-well plate format, the results from a large number of samples can be analyzed in 2 hr. A software program, Primer Express™, was used for designing primers.

2. Optimize DNA/RNA extraction protocols

We compared three commercial kits for extraction of DNA and RNA from shrimp tissues. For DNA extraction, the QIAamp DNA Mini kit (Qiagen, CA, USA) yielded the highest amounts of total DNA. The DNA solutions extracted from various shrimp tissues contained 20-48% ds (double-stranded) DNA. The yield of ds DNA per mg of tissue was also highest with this kit, ranging from 0.14 to 0.82 ：g of DNA per mg of tissue. For RNA extraction, use of the RNeasy kit (Qiagen, CA, USA)

produced the highest yields. The yield of RNA was 0.136-0.162 ：g per mg of tissue, and the quantity of DNA contamination was low, ranging from 3.3 to 13.9 ng/：l.

3. Optimize real-time PCR

The concentrations of the primers for real-time PCR (WSSV and IHHNV) were optimized by testing three concentrations: 50, 300, and 900 nM. The data showed that 300 nM of primer appeared to be the optimum concentration that yielded the lowest threshold cycle (Ct) and maximum magnitude of fluorescent signal ( ) Rn). The amount of template DNA needed was optimized by testing 10, 50 and 100 ng of DNA in a real-time IHHNV PCR. The results indicated that a 10 ng DNA template yielded the highest signal. Thus, 10 ng of template DNA was used for real-time PCR assays.

The reaction mixture included a TaqMan Universal PCR master Mix (Applied Biosystems, Foster City, CA), primers (300 nM each), a TaqMan probe (150 nM), and extracted total DNA as a template with the following profile: 2 min at 50ºC and 10 min at 95ºC, 40 cycles of 15 s at 95ºC and 1 min at 60ºC. The reactions were carried out in a real-time PCR instrument, GeneAmp 5700. The Ct value is 40 when using specific-pathogen-free (SPF) shrimp DNA as a negative control. The Ct values were less than 40 when virus infected samples were used as templates.

4. Optimize real-time RT-PCR

Initially, SYBR Green chemistry was used for the detection of both YHV and TSV in real-time RT-PCR. The data indicated that high levels of primer-dimers were formed in the non-template control. This generated a false positive if determined only by Ct value. Optimization of the primer-dimers proved difficult even with several attempts to adjust concentrations and ratios of the primers. The sequence of the primer appears to be the one of most important factors for dimer formation.

Later, TaqMan chemistry was used, and this was found to be a better choice as a diagnostic tool. There is no signal detected with either of the negative controls: non-template control and specific-pathogen-free shrimp RNA. The primer concentration was optimized to be 300 nM. The probe concentration was determined by testing various concentration (50, 100, 150, 200 and 250 nM), and the results showed that 100, 150, and 200 nM were satisfactory. A concentration of 100 nM was used to reduce the cost. The TaqMan one-step RT-PCR master Mix (Applied Biosystems) was used for RT-PCR. The cycling consisted of 48ºC, 30 min for reverse transcription and 40 cycles of 95ºC for 15 s and 60ºC for 1 min.

5. Detection and quantification of virus through real-time PCR (and RT-PCR)

A. WSSV

Selection of PCR primers/TaqMan probes for WSSV

A set of PCR primers and TaqMan probe for WSSV detection were selected from a WSSV genomic sequence deposited in GenBank (accession no. U50923). These primers generate a 69-bp amplicon.

Primers/probe	Nucleotide sequence
WSSV1011F	5'-TGG TCC CGT CCT CAT CTC AG-3'
WSSV1079R	5'-GCT GCC TTG CCG GAA ATT A-3'
WSSVTaqMan	5'-AGC CAT GAA GAA TGC CGT CTA TCA CAC A-3'

Specificity and sensitivity of real-time WSSV PCR

Specificity of this assay was determined using various viruses as templates in a real-time PCR. The results showed that the Ct values are 40 for shrimp DNA, other baculoviruses (BP and MBV), and two shrimp parvoviruses (IHHNV, HPV). The primers react to various WSSV isolates (China, South Carolina, Texas, India, crayfish), and generate strong amplification signals from these samples. The 69-bp WSSV amplicon generated from real-time PCR was cloned and designated as pWSSV-1. The sensitivity is determined to be 4 copies when using a series of concentrations of the positive standard (Fig.1).

Quantification of WSSV in infected shrimp

Juvenile *P. stylirostris* that were moribund from a *per os* experimental infection contained 1.3 x 10^7 to 9.0 x 10^{10} copies of WSSV in the pleopod samples (Table 1). *P. monodon* collected from Indonesia were also detected with WSSV infection at a level of 2.1 x 10^6 copies/：g DNA.

Various tissues of infected *P. vannamei* were analyzed for their quantities of WSSV. The results showed that hemolymph contained the highest level of WSSV when compared to other tissues (Table 2). There were 2.5 x 10^9 copies of WSSV/：g DNA detected in hemolymph; this is significantly higher than gills and pleopods. Pleopods and gills had a mean of 1.6 x 10^9 and 1.2 x 10^9 copies of WSSV/：g DNA, respectively. There appears no statistically significant difference of viral load between these two tissues. Pleopods and gills contained statistically (95% confidence) more WSSV than muscle (1.9 x 10^8 copies/：g DNA) and hepatopancreas (9.0 x 10^7 copies/：g DNA).

Another set of PCR primers and TaqMan probe for WSSV detection were selected from the genomic sequence of ribonucleotide reductase large unit (rr1, GenBank AF369029). These primers generate a 68-bp amplicon. A DNA fragment containing a portion of rr1 gene (506 bp, nucleotide 830 to 1,335) was cloned and designated as pWSSV-2. This set of primers/probe is used as a confirmatory assay.

Primers/probe	Nucleotide sequence
WSSVrr1-981F	5'-GCA TCT GGA CGT GAA GGA CTT A-3'
WSSVrr1-1056R	5'-GAA AAG ATC CCT CGT CCT CAA A-3'
WSSVprobe-rr1	5'-TGA CTG CAG AAA GAA TGC CGG TAA TGA AG-3'

B. IHHNV

Selection of PCR primers/TaqMan probes for IHHNV

In order to detect IHHNV isolated from various geographic regions, a conserved region of the IHHNV genome was selected to permit binding of both the PCR primers and the TaqMan probe. ORF1, which encodes for the non-structural protein, was chosen as the target region. The sequences of primers (IHHNV1608F/1688R) and the internal TaqMan probe used were determined with Primer Express™ software, and these were used to amplify a 81-bp amplicon in a real-time PCR. The target sequence is from nucleotide 1,608 to 1,688 within the IHHNV genome (GenBank AF218266).

Primers/probe	Nucleotide sequence
IHHNV1608F	5'-TAC TCC GGA CAC CCA ACC A-3'
IHHNV1688R	5'-GGC TCT GGC AGC AAA GGT AA-3'
IHHNVTaqMan	5'-ACC AGA CAT AGA GCT ACA ATC CTC GCC TAT TTG-3'

Specificity of real-time IHHNV PCR

To determine if this real-time IHHNV PCR assay can be used to detect IHHNV DNA, it was first applied for the detection of CsCl-purified IHHNV (Hawaii isolate) virions. The result was positive and the Ct value was 13.28 (Fig. 2). Detection with the total DNA extracted from IHHNV-infected *P. stylirostris* (Mexico isolate) was also positive and the Ct value was 17.57. The real-time PCR appears to be specific to IHHNV DNA; it did not detect HPV virions, DNA extracted from WSSV-infected *P. vannamei,* or DNA extracted from SPF *P. vannamei*. The Ct values for these samples were 40. These results were confirmed by observing the real-time PCR products after agarose gel electrophoresis.

Sensitivity of real-time IHHNV PCR

To determine the sensitivity of real-time IHHNV PCR, a DNA fragment containing 757 bp of IHHNV non-structural protein (nucleotide 1,215 to 1,971) was cloned in the pGEM-T-Easy vector, designated as pIHHNV-P4, and used as a positive standard. The concentration was determined and expressed as number of molecules per reaction. By testing from 5 to 5 x 10^7 copies of pIHHNV-P4 plasmid, we determined that the limit of detection of the assay is 10 copies of IHHNV DNA (Fig. 3). The average coefficient of regression of the standard curve was 0.99.

Quantification of IHHNV in infected shrimp

The real-time PCR assay was used to quantify IHHNV in infected shrimp collected from various locations (Mexico, Guam, the Philippines, and Belize) and from laboratory challenge studies. The quantitative analysis showed that wild-caught, large juvenile *P. stylirostris,* collected from the Gulf of California (Mexico) in 1996, were naturally infected with IHHNV and contained up to 10^9 copies of IHHNV per ：g of DNA. Similar quantities of IHHNV were detected in hatchery-raised, small juveniles of *P. stylirostris* collected from Guam in 1995 and in farm-raised post-larval *P. monodon*

from the Philippines in 1996. Laboratory infected *P. stylirostris* contained approximately 10^8 copies of IHHNV 31 days after being fed with IHHNV-infected shrimp tissue. We also confirmed that samples of Super Shrimp®, a line of *P. stylirostris* selected for IHHNV resistance, showed no signs of infection 32 d after ingesting IHHNV-infected shrimp tissue. A level of 10^9 copies of IHHNV was detected in the farm-raised *P. vannamei* showing runt-deformity syndrome collected from Belize in 2001 (Table 2).

C. TSV

Selection of RT-PCR primers/TaqMan probes for TSV

A set of RT-PCR primers and TaqMan probe were selected form the region of ORF1 that encodes non-structural proteins of TSV (GenBank AF277675, Mari and Lightner, unpublished data). These primers amplify a 72-bp DNA fragment after RT-PCR.

Primers/probe	Nucleotide sequence
TSV1003F	5'-TTG GGC ACC AAA CGA CAT T-3'
TSV1074R	5'-GGG AGC TTA AAC TGG ACA CAC TGT-3'
TSV-Probe-1	5'-CAG CAC TGA CGC ACA ATA TTC GAG CAT C-3'

Sensitivity of real-time TSV RT-PCR

To determine the sensitivity of real-time TSV RT-PCR, a 0.4 kb TSV genomic insert containing its target sequence was inserted into a plamsid (pGEM-T Easy). The resulting plasmid, designated as pTSV-rt1, was purified and linearized at a unique PstI site downstream from the end of the inserted fragment, and was used as template DNA (1 ：g of plasmid per 20 ：l reaction) in an in vitro transcription reaction with T7 RNA polymerase at 37°C for 2 hr. Following the reaction, template DNA was digested with DNase I, the RNA (503 nucleotides) was recovered by phenol-chloroform extraction and ethanol precipitation. The RNA was re-suspended in diethylpyrocarbonate (DEPC)-treated water and quantified by a spectrophotometer. This TSV RNA was diluted from 10 to 10^8 copies and used as a template in a real-time RT-PCR. The detection limit was 10 copies (Fig. 4), the non-template control gave a negative result. The amplification showed a linear relationship over eight 10-fold dilutions.

Quantification of TSV in infected shrimp

Juvenile *P. vannamei* (25 shrimp) were fed with TSV-infected tissues for 3 days at a rate of 5% total body weight. Shrimp started to die 4 days after exposure to TSV and only 5 shrimp survived 30 days after feeding (Fig. 5). Total RNA was extracted from gill tissues of moribund shrimp (4 shrimp) and surviving shrimp (2 shrimp) and amplified along with a serial dilution of the standard. The shrimp collected between days 4 and 8 contained 1.4×10^7 copies of TSV/：g RNA. The surviving shrimp had 70 times less TSV RNA, 2.0×10^5 copies/：g RNA (Fig. 5).

A second set of primers/probe was selected and used for confirmation, these TSV-HSE-P1/P2 amplify a 97-bp RNA fragment.

Primers/probe	Nucleotide sequence
TSV-HSE-P1	5'-GCG CCT ACG TGT CCG AAA T-3'
TSV-HSE-P2	5'-CAC ATC CGC AGC GGA TAT C-3'
TSV-Probe-HSE	5'-TGA TAT TAG GCG AGT TGC GCG CCA-3'

D. YHV

Selection of PCR primers/TaqMan probes for YHV

The RT-PCR primers and TaqMan probe were selected from a portion of putative RNA polymerase gene of YHV (GenBank AF148846), and they generate a 66-bp amplicon.

Primers/probe	Nucleotide sequence
YHV141F	5'-CGT CCC GGC AAT TGT GAT C-3'
YHV206R	5'-CCA GTG ACG TTC GAT GCA ATA-3'
YHVTaqMan	5'-CCA TCA AAG CTC TCA ACG CCG TCA-3'

A YHV clone, designated as 3-27, containing a 1.1 kb YHV genomic insert was purified, linearized, and used as a template in an in vitro transcription reaction. A 760 nucleotide of YHV RNA was generated as a positive control for real-time RT-PCR.

6. Evaluation of sensitivity and reproducibility of real-time PCR and RT-PCR

To determine the sensitivity of each real-time PCR (and RT-PCR) assay, a portion of each viral genome was cloned. The resulting plasmid with the viral insert was used as a standard. The concentrations were determined and expressed as number of molecules per reaction. By testing from 1 to 10^8 copies of each positive control, we determined that the limit of detection of real-time PCR is 1-10 copies for IHHNV DNA and WSSV DNA (Fig. 1 and 3). This is more sensitive than traditional PCR, which is 3-6 x 10^4 copies/：g DNA, as determined in our laboratory.

Evaluation

The objectives of this project have been achieved. This includes (1) the purchase and installation of a real-time PCR detection system at University of Arizona, (2) development of real-time PCR (and RT-PCR) detection protocols for WSSV, IHHNV, TSV, and YHV, and (3) comparison of the results of real-time PCR with our existing, traditional PCR procedures.

Dissemination of project results

The project results have been made available to the public via several publications and

presentations:

1. Tang KFJ and Lightner DV (2001) Detection and quantification of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in penaeid shrimp by real-time PCR. Global Aquaculture Advocate 4(5): 20-21.

2. Lightner, DV, Durand SV, Redman RM, Mohney LL, Tang KFJ (2001) Qualitative and quantitative studies on the relative virus load of tails and heads of shrimp acutely infected with WSSV: implications for risk assessment. Pages 285-291. *In*: Browdy CL and Jory DE (editors). The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture, Aquaculture 2001. The World Aquaculture Society, Baton Rouge, LA USA.

3. Tang KFJ. and Lightner DV (2001) Detection and quantification of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp by real-time PCR. Dis. Aquat. Org. 44:79-85..

4. Lightner DV, Durand SV, Redman RM, Mohney LL, Tang KFJ (2000) Viral load in heads and tails: study reveals risks in WSSV-infected shrimp. Global Aquaculture Advocate 3(6) 13-15.

Two papers were presented in World Aquaculture Society annual meeting (2001) held in Lake Buena Vista, Florida

1. Lightner DV, Tang KFJ, Durand SV, Redman RM, Mohney (2001) Qualitative and quantitative studies on the relative virus load of tails and heads of shrimp acutely infected with WSSV. pp. 374.

2. Tang KFJ and Lightner DV (2001) Detection and quantification of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in penaeid shrimp by real-time PCR. pp. 635.

Two additional manuscripts based on this work are currently being prepared:

1. Durand SV and Lightner DV. Quantitative real-time PCR for the measurement of white spot syndrome virus in shrimp.

2. Tang KFJ, Wang J and Lightner DV. Development of a real-time quantitative RT-PCR for Taura syndrome virus.

These techniques have been used as one of the diagnostic assays for shrimp viruses at the University of Arizona.

Table 1. Quantitative analysis of WSSV by real-time PCR.

Sample no.	WSSV	Species	Origin	Tissues	Copy no. : g^{-1} DNA
1	Thailand	*P. stylirostris* (SuperShrimp®)	lab infection	pleopods	3.0×10^{10}
2	Indonesia	*P. monodon*	farm	pleopods	2.1×10^{6}
3	Thailand	*P. stylirostris* (SuperShrimp®)	lab infection	whole PLs	4.3×10^{9}

Table 2. Mean number of WSSV genome present in pleopods, gills, hepatopancreas, muscle and hemolymph.

Samples	n	Mean	Std deviation	Range
Pleopods	5	$1.60x10^9$	$6.24x10^8$	$7.5x10^8 – 2.50x10^9$
Gills	5	$1.2x10^9$	$8.5x10^8$	$4.8x10^8 – 2.6x10^9$
Hepatopancreas	5	$9.0x10^7$	$6.0x10^7$	$3.5x10^7 – 1.9x10^8$
Muscle	5	$1.9x10^8$	$1.3x\ 10^8$	$5.2x10^7 – 3.4x10^8$
Hemolymph	3	$2.55x10^9$	$3.5x10^9$	$2.15x\ 10^9 – 2.80x10^9$

Table 3. Quantitative analysis of IHHNV by real-time PCR. (p.f.= post feeding).

Sample no.	IHHNV	Species	Origin	Tissues	Copy no. : g^{-1} DNA
1	Mexico	*P. stylirostris*	wild-caught	gills	1.1×10^9
				pleopods	4.2×10^8
2	Guam	*P. stylirostris*	hatchery	heads	1.2×10^9
3	Philippines	*P. monodon*	farm	whole PLs	1.6×10^9
4	Mexico	*P. stylirostris*	lab infection, day 31 p.f.	pleopods	2.2×10^8
5	Panama	*P. stylirostris* (Super Shrimp®)	lab infection. day 32 p.f.	heads	not detected
6	Belize	*P. vannamei*	farm	pleopods	4.5×10^9

Figure 1. Standard curve of the WSSV copy number versus Ct (threshold cycle) value.

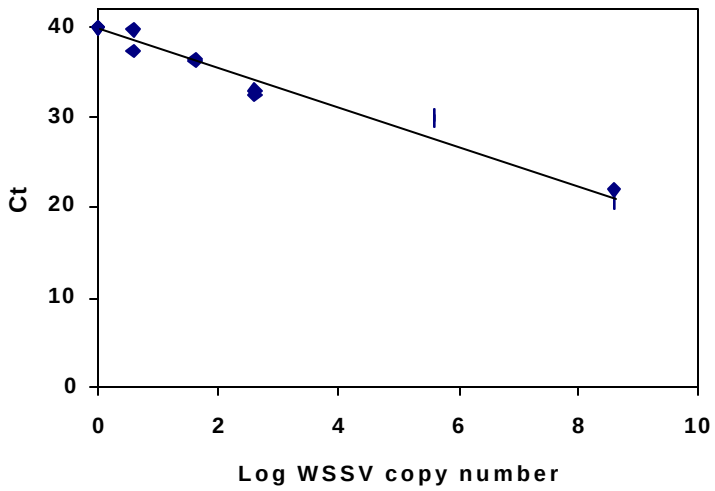

Figure 2. Amplification plots for CsCl-purified IHHNV virions (green) and extracted IHHNV DNA (red). Rn (normalized reporter) is the emission of the reporter. The Rn between cycle 6 and 11, value of 0.03, was set as background fluorescence in this experiment.

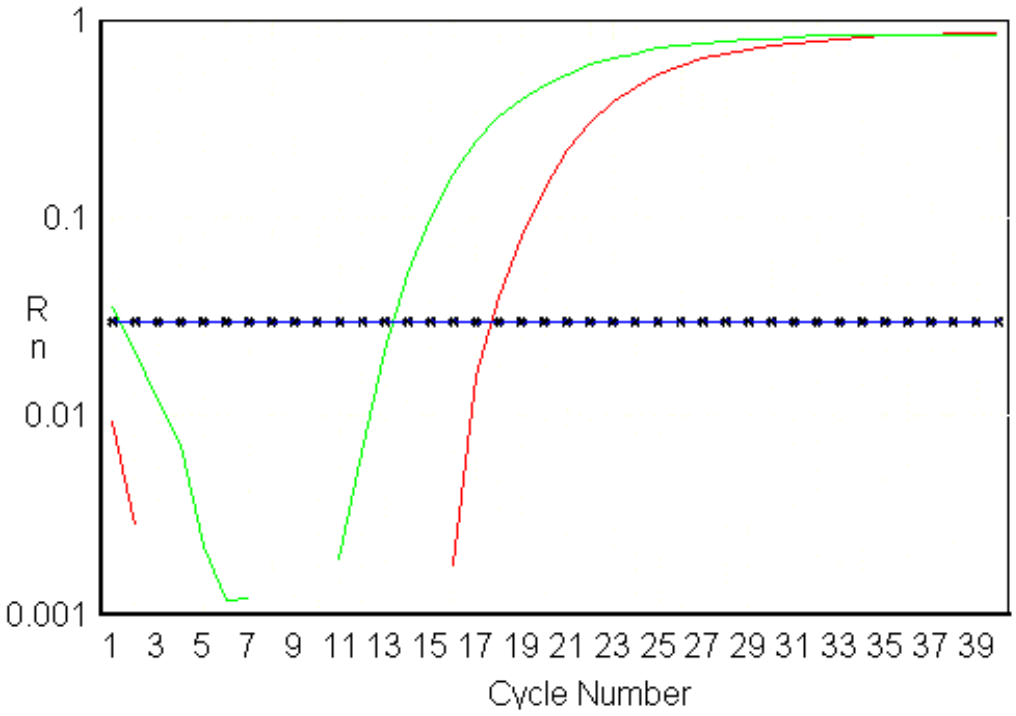

Figure 3. Standard curve of the IHHNV copy number versus Ct value. Purified recombinant plasmid was serially diluted from 5 x 10^7 to 5 copies used as template in real-time PCR. The resulting Ct values are plotted against the logarithm of their respective copy numbers.

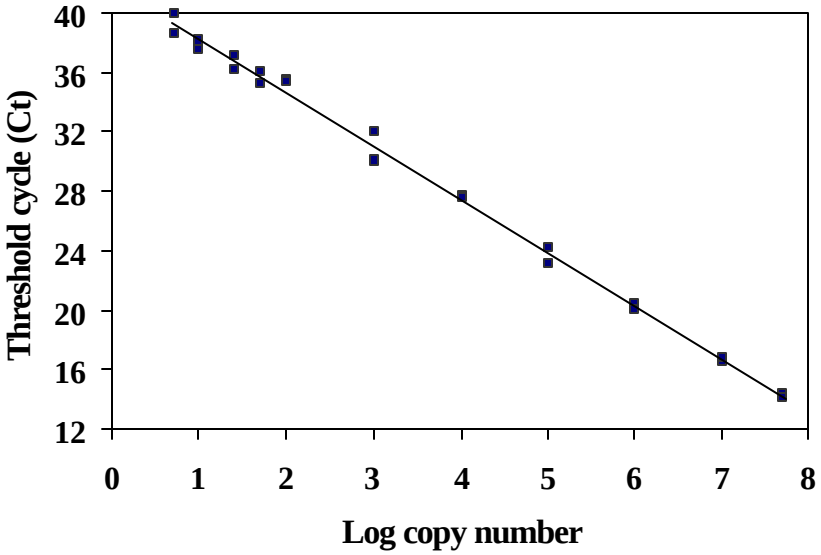

Figure 4. Standard curve of the TSV copy number versus Ct value.

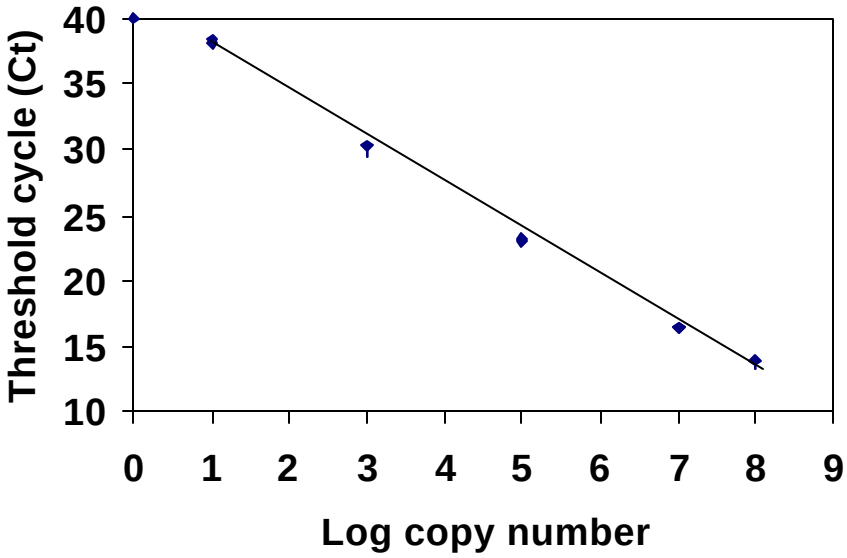

Fig 5. Quantitative analysis of TSV-infected shrimp in acute phase and chronic phase.